Genetic comparison between Pinus sylvestris and P. mugo using isozymes and chloroplast DNA

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Pinus sylvestris and *P. mugo* populations from Poland and Czechoslovakia were compared using genetic variability at isozyme markers, chloroplast DNA variation, and mating system measurements. Two isozyme loci were found to differ between the species. *P. mugo* was as variable at isozyme loci as *P. sylvestris*. Diagnostic cpDNA fragments were found using the restriction enzyme *Bcl–I*. Populations that were morphologically classified as hybrids were found to be pure species, based both on isozyme and cpDNA results.

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Introduction

Among the about 100 species of the genus *Pinus* there are many species pairs or groups of species that are very closely related to each other (Mirov 1967). Many of these species can hybridize with each other, and sometimes it may be difficult to distinguish the pure species and the putative hybrids. The *Pinus mugo* Turra complex is closely related to *P. sylvestris* L. Both are in the subgen. *Pinus*, sect. *Pinus*, subsect. *Sylvestres* Loud. emend Little & Critchfield (see Christensen 1987a for a recent taxonomic revision). *P. mugo* is a complex endemic to southern and central Europe, where it is sympatric with *P. sylvestris*. The two subspecies of *P. mugo*, the eastern ssp. *mugo* and the western ssp. *uncinata* are sympatric in the Alps, the lowlands of central Europe, and the northwestern Carpathians. The two species differ morphologically in growth form, bark, shape of the cross section of the needles and the distances between resin ducts, and the angle of the conelets (Marcet 1967; Christensen 1987a). It has long been supposed on morphological grounds that hybridization occurs between *P. sylvestris* and *P. mugo* in the Alps (Marcet 1967; Neet-Sarqueda et al. 1988) and in the Carpathians. Many studies have been conducted on the morphological variability of the species and their supposed hybrids in Poland and Czechoslovakia (Staszkiewicz & Tyszkiewicz 1969, 1972). Prus-Glowacki & Szweykowski (1983) have studied some isozyme loci, Prus-Glowacki et al. (1981) immunological reactions.

Here we examine genetic variability at a large number of isozyme loci and chloroplast DNA and evaluate the differences between species. Chloroplast DNA has proved to be diagnostic between closely related species in many cases, and has been a valuable aid in studies of hybridization, e.g. because of its uniparental mode of inheritance. In conifers, cpDNA has been found to be paternally inherited, and it has been successfully used in many studies of hybridization (Wagner et al. 1987; Wang & Szmidt 1990). Further, we measured the outcrossing rate in both species. Finally, we have included in the study four populations that were classified as hybrids on a morphological basis, and we use our genetic markers to evaluate the status of these populations.

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Tab. 1. Populations, their abbreviations, locations, altitudes, and material used.

Species	Abbreviation	Location	Altitude (m)	Material
P. sylvestris (1)	Sylv1	50°00′–50°10′N 15°50′–16°10′E	250	Seeds, needles
P. sylvestris (2)	Sylv2	49°20′–49°50′N 15°–16°E	600–700	Seeds
P. mugo (3)	Mugo1	50°45′N 15°27′E	1240	Needles
P. mugo (4)	Mugo2	49°11′N 19°48′E	1400	Seeds
P. mugo (5)	Mugo3	49°11′N 20°00′E	1400	Seeds
P. sylvestris (6) (suspected hybrid with *P. mugo*)	H1	50°02′N 12°49′E	800	Seeds, needles
P. sylvestris (7) (suspected hybrid with *P. mugo*)	H2	50°10′–50°30′N 12°20′–13°00′E	500–600	Seeds, needles
P. sylvestris (8) (suspected hybrid with *P. mugo*)	H3	48°59′N 13°48′E	750–8090	Seeds, needles
P. mugo (9) (suspected hybrid with *P. sylvestris*)	H4	40°04′N 20°03′E	110–1200	Seeds

Material and methods

The populations, their abbreviations, locations, and altitudes, and material used are presented in Tab. 1, a map is shown in Fig. 1. All the populations are located in Czechoslovakia except H2, which grows in Germany. The population Mugo1 originates from the French Alps but has been transferred to Czechoslovakia. The seeds were provided to us by Dr Kanak of the Czechoslovakian Forest Service.

Seeds were collected from the sites listed in Tab. 1 and needles were collected from six month old seedlings grown from the seeds in a greenhouse.

Chloroplast DNA (cpDNA) was extracted from needles from the populations Sylv1, Mugo1, H1, H2, and H3 as described by Szmidt et al. (1986) and White (1987). All DNA samples were extracted from bulk samples of several seedlings of a population. DNA samples (1 µg) were digested for 4 hours with 10 units of restriction enzyme *Bcl–I*, which was found to detect species specific restriction fragments. Electrophoresis was carried out in 0.7% agarose gel for 16 hours at 3V/cm in TBE buffer in the presence of ethidium bromide (Sambrook et al. 1989). BRL 1 kb ladder was used as a molecular weight standard. The gel was photographed in UV light using 665 Polaroid film. The lengths of fragments were determined by the method of Schaffer & Sederoff (1981). Restriction fragment patterns of the suspected hybrid populations H1, H2, and H3 were compared with those of pure species (populations Sylv1 and Mugo1).

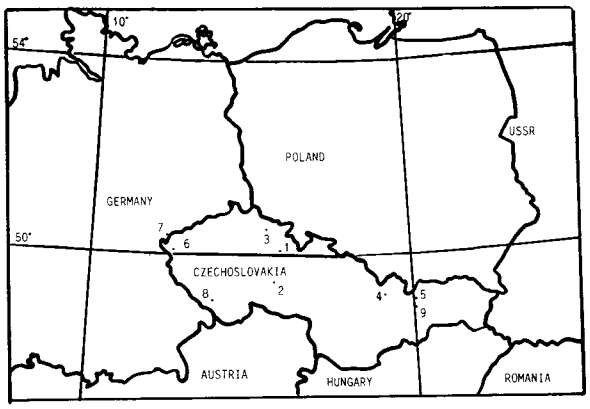

Fig. 1. Populations *Pinus sylvestris* and *P. mugo*.

The embryo and the megagametophyte of seeds were analysed by starch gel electrophoresis. In total 10 loci were analysed. The enzymes studied were fluorescent esterase (F-Est, E.C.3.1.1.1), glutamate dehydrogenase (GDH, E.C.1.4.1.3), glutamateoxaloacetate-transaminase (GOT, E.C.2.6.1.1), malate dehydrogenase (MDH, E.C.1.1.1.37), 6-phosphogluconate dehydrogenase (6-PGD, E.C.1.1.1.44), and shikimate dehydrogenase (SDH, E.C.1.1.1.25). The references for the methods of electrophoresis and staining are listed by e.g. by Muona et al. (1988). The most anodally migrating band at each locus, and the corresponding allele, was named 1.

The genotype of the megagametophyte equals that of the egg cell. Hence, the genotypes of megagametophytes and embryos in a seed allow deducing the genotype of the pollen grain. Allelic frequencies between ovule and pollen pools within all the populations were compared with G-tests. Fixation indices measure the deviation of genotypic frequencies from panmictic

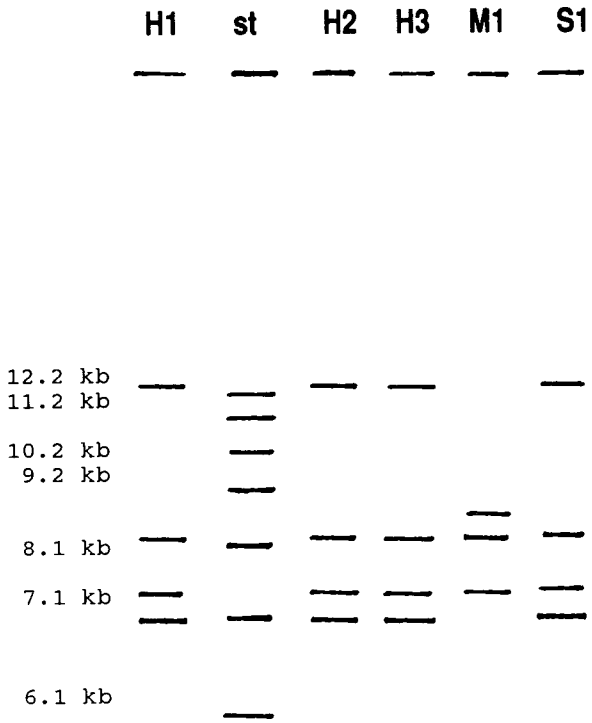

Fig. 2. Schematic drawing illustrating cpDNA restriction patterns from the populations Sylv1, Mugo1, H1, H2, and H3 generated by *Bcl*-I. Lane 1: H1, Lane 2: 1 kb ladder, Lane 3: H2, Lane 4: H3, Lane 5: Mugo1, Lane 6: Sylv1.

Hardy-Weinberg expectation. Positive fixation indices suggest selfing or other inbreeding. Fixation indices and their variances were estimated (Brown 1970). Outcrossing rates for those populations, where single tree progenies were available (Sylv2, Mugo2, Mugo3, and H4) were estimated using the multilocus method for conifers of Ritland & El-Kassaby (1985). Nei's genetic distances (Nei 1972) between all pairs of populations were estimated. A dendrogram was constructed from the matrix with average distance method (Nei 1987: 293).

Results

cpDNA

Chloroplast DNA was contaminated with nuclear DNA, which gave heavy background to the cpDNA bands. Thus only the largest cpDNA fragments could be reliably detected. The largest restriction fragments of cpDNA digested with *Bcl–I* from populations Sylv1, Mugo1, H1, H2, and H3 are shown in Figs 2 and 3. The fragment lengths are presented in Tab. 2. Among the largest fragments, the 8.8 kb fragment was found only in *P. mugo*. *P. mugo* lacks the 7.1 kb fragment present in *P. sylvestris*. There were other differences in the smaller size classes, but even the one 8.8 kb fragment suffices for classifying the species. The cpDNA of the suspected hybrid populations H1, H2, and H3 was identical to the cpDNA from the pure *P. sylvestris* population. Thus the pollen to these seedlots was from *P. sylvestris*.

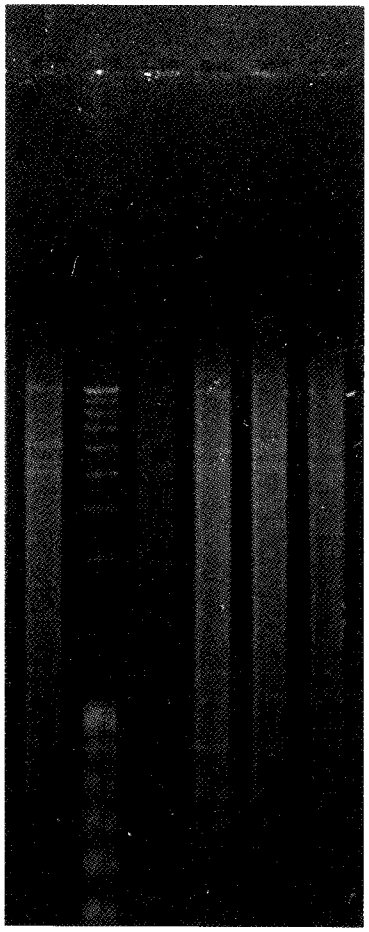

Fig. 3. Restriction fragments of cpDNA after digestion with *Bcl–I*. Lanes as in Fig. 2.

Isozymes

The frequencies of the most common alleles of each population are presented in Tab. 3. At the locus *Mdh–3* the most common allele of the pure *P. sylvestris* pop-

Tab. 2. Lengths (bp) of the largest fragments of cpDNA from the populations Sylv1, Mugo1, H1, H2 and H3 digested with *Bcl–I*.

Sylv1	Mugo1	H1	H2	H3
12 400	8 800	12 400	12 400	12 400
8 400	8 400	8 400	8 400	8 400
7 500	7 500	7 500	7 500	7 500
7 100		7 100	7 100	7 100

Tab. 3. Frequencies of the most common alleles at 10 loci in 8 populations of *Pinus sylvestris*, *P. mugo*, and their suspected hybrids.

Locus	Allele	Sylv1	Sylv2	Mugo2	Mugo3	H1	H2	H3	H4
F-Est	1	0.69	0.64	0.85	0.90	0.83	0.76	0.79	0.97
Gdh	1								0.50
	2	0.71	0.68	0.49	0.49	0.65	0.77	0.67	
Got-1	2	0.99	1.00	0.99	1.00	1.00	1.00	1.00	1.00
Got-2	5	0.63	0.64	0.73	0.87	0.75	0.73	0.61	0.59
Mdh-1	2	0.99	0.97	1.00	1.00	1.00	1.00	0.95	1.00
Mdh-2	1	0.99	0.95	0.84	0.75	0.87	1.00	1.00	0.98
Mdh-3	1	0.75	0.75			0.61	0.67	0.86	
	2			0.87	0.78				0.74
6-Pgd-2	1	0.70	0.63			0.81	0.71	0.85	
	2			0.92	0.67				0.96
Sdh-1	2	0.80	0.91			0.77	0.87	0.72	0.48
	3			0.60	0.62				
Sdh-2	3	0.94	1.00	1.00	1.00	1.00	0.99	1.00	0.85
N		100	81	120	230	101	102	112	204

ulations and the suspected hybrid populations H1, H2, and H3 was allele number 1, whereas in pure *P. mugo* populations and in the suspected hybrid population H4 the most frequent allele was number 2. The populations differed in a similar way at the locus *6–Pgd–2*. At the locus *Sdh–1* the most common allele in the pure *P. sylvestris* populations and all the suspected hybrid populations was allele number 2, whereas in pure *P. mugo* populations the most frequent allele was number 3. At the rest of the loci studied all the populations shared the same most frequent alleles. Allelic frequencies in the ovule and pollen pools differed from each other only at a few loci in each population (data not shown). Average expected heterozygosity in the two *P. sylvestris* populations was 0.27 and 0.26, in both *P. mugo* populations 0.24.

Average fixation indices are presented in Tab. 4. They did not differ significantly from zero, and suggest at most low levels of inbreeding.

Outcrossing rates (t) for the populations Sylv2, Mugo2, Mugo3, and H4 are presented in Tab. 5. The loci used were *Gdh*, *Mdh–2*, *Mdh–3*, *6–Pgd–2*, and *Sdh–1*. The average outcrossing rate for *P. mugo* was 0.85, the *P. sylvestris* population had a fairly low estimate of 0.90, and the hybrid population 0.92.

Tab. 4. Average fixation indices (F) and their standard deviations (sd) in populations of *Pinus sylvestris* and *P. mugo*.

Population	F	sd
Sylv1	−0.02	0.01
Sylv2	−0.03	0.01
Mugo2	−0.01	0.01
Mugo3	−0.01	0.01
H1	−0.01	0.01
H2	−0.01	0.01
H3	−0.01	0.01
H4	−0.01	0.01

Genetic distances between the populations are presented in Tab. 6. The average distance between pure *P. mugo* and pure *P. sylvestris* was 0.14. The average distance between pure *P. sylvestris* populations and the suspected hybrid populations H1, H2, and H3 was 0.01 and between pure *P. sylvestris* populations and the population H4 0.13. The average genetic distance between pure *P. mugo* populations and the populations H1, H2, and H3 was 0.13 and between the pure *P. mugo* population and the suspected hybrid population H4 0.04. Fig. 4 shows a dendrogram that further clarifies the relationships of the populations.

Discussion

Pinus mugo and *P. sylvestris* are typical conifers in having high levels of genetic variability. *P. sylvestris* is known to be among the most variable species (Muona 1989), and *P. mugo* has similar levels of variability. The outcrossing rates here were lower than average for the genus *Pinus* (see Muona 1989). *P. sylvestris* has in most studies had a higher level of outcrossing. The estimate for *P. mugo* is among the lowest in the genus. Thus there seems to be some partial selfing. However, the fixation indices did not detect the inbreeding.

Tab. 5. Multilocus outcrossing estimates (t) and their standard deviations for *Pinus sylvestris* (Sylv2), *P. mugo* (Mugo2, Mugo3) and a suspected hybrid population (H4).

Population	t	sd
Sylv2	0.90	0.09
Mugo2	0.93	0.08
Mugo3	0.78	0.04
H4	0.92	0.04

Tab. 6. Nei's genetic distances between populations of *Pinus sylvestris* (Sylv), *P. mugo*, and their suspected hybrids (H).

Populations	Sylv1	Sylv2	Mugo2	Mugo3	H1	H2	H3
Sylv2	0.005						
Mugo2	0.155	0.153					
Mugo3	0.113	0.117	0.021				
H1	0.011	0.015	0.133	0.084			
H2	0.005	0.005	0.145	0.099	0.008		
H3	0.008	0.015	0.189	0.136	0.013	0.014	
H4	0.130	0.131	0.022	0.056	0.127	0.128	0.165

Tab. 6 and Fig. 4 give an unequivocal result on the relationship between the populations. They form two distinct groups, which are internally quite homogeneous. Populations H1, H2, and H3 are grouped together with the *P. sylvestris* populations. The suspected hybrid populations differ hardly more from the pure species than the two *P. sylvestris* among themselves. This low differentiation is typical of conifers (e.g. Muona 1989). The suspected hybrid population H4 is grouped close to the two *P. mugo* populations. The distance between the two groups in the dendrogram was 0.145. These can be compared to the review of Nei (1976), who has estimated that genetic distances between species are 0.1–2.0 and between subspecies 0.02–0.2, as measured with allozyme loci. Clearly the differences between *P. sylvestris* and *P. mugo* are at the very low end of this range. This low differentiation would account for the variable views on the morhology and taxonomy of the two species (see introduction and e.g. Mirov 1967). The low level of differentiation is also concordant with the ability of the species to be crossed artificially.

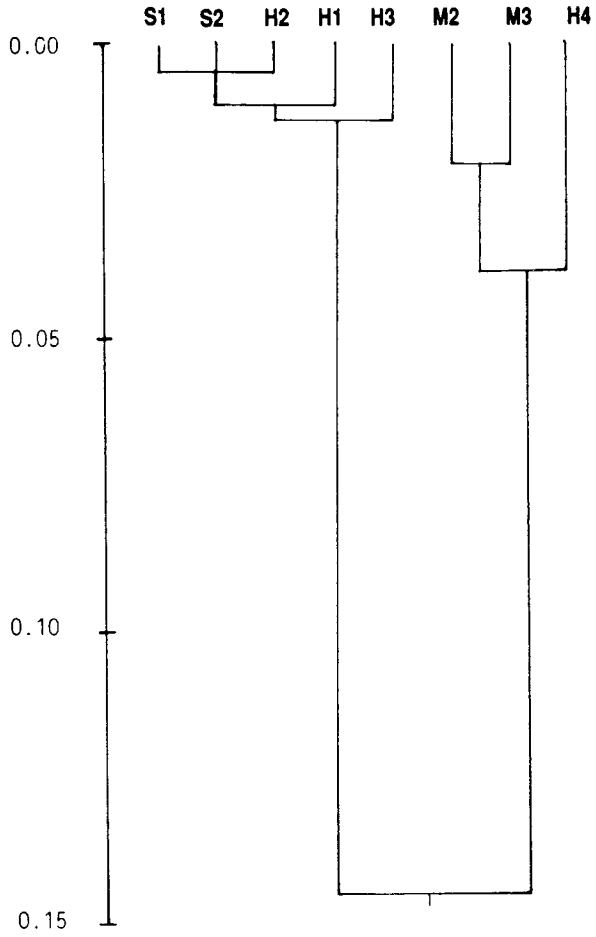

Fig. 4. Dendrogram of populations constructed from the distances in Tab. 6 with the unweighted pair-group method with arithmetic mean. S – *Pinus sylvestris*, M – *Pinus mugo*, and H – hybrid populations.

The restriction fragment patterns of the suspected hybrid populations H1, H2, and H3 were identical to the restriction patterns of pure *P. sylvestris*. The fragment of 8.8 kb which is typical of *P. mugo*, could not be found in any of them. This indicates that these populations have not received any pollen from *P. mugo*.

Except at few loci, the allelic frequencies in the ovule and pollen pools did not differ from each other in the four suspected hybrid populations. Thus both ovules and pollen represent the same gene pool. We conclude that these populations have not received pollen from the other species.

The results given by cpDNA and isozyme data analysis do not give any proof of hybridization in the putative hybrid populations. This kind of contradiction between morphological classification and and other methods has been found before by Rieseberg et al. (1988) when studying different races of *Helianthus bolanderi*. It had earlier been assumed that a weedy race of this species originated through recent introgression. However, detailed molecular studies showed that the race was of ancient origin. Szmidt et al. (1988) classified seedlots *Picea glauca* and *Picea sitchensis* using cpDNA. There were two seedlots classified as hybrids based on morphology, but only one proved to have cpDNA from both parental species.

The many studies on morphological variation of the two pines have often found intermediate forms between the species. Staszkiewicz & Tyszkiewicz (1969) classified 22 trees as *P. sylvestris* and the rest as hybrids in a Carpathian population. Later they considered many other populations as hybrid populations (Staszkiewicz

& Tyskiewicz 1972). Hybridization can potentially occur between the species. It has been demonstrated with artificial crosses (Dengler 1932). However, the views on the frequency of hybridization differ. Christensen (1987b), based on his morphological studies, was of the opinion that hybridization is rare. The above mentioned studies in Poland suggest that hybrids are more frequent. Prus-Glowacki & Szeykowski (1983) concluded that hybrid trees resembled *P. mugo* with respect to peroxidases, *P. sylvestris* with respect to esterases. In a stable hybrid, one would expect to see intermediate allelic frequencies at most loci, as has been found in *P. densata* (Wang et al. 1990).

The phenotype of an organism is a result of the interaction between different genes and environment. In different environments the same genotype can produce different phenotypes and the same phenotype can result from different gene combinations (Clausen et al. 1940; Falkenhagen & Nash 1978). Because the suspected hybrid populations H1, H2, and H3 proved to be pure *P. sylvestris* populations, their exceptional morphology must be due to environmental variation. Our data lend thus support to Christensen's (1987b) conclusion that hybridization may be rare. A corollary of this conclusion would be that the morphological intraspecific variability is higher than has been suspected. In the future, the importance of hybridization between these two species should be clarified by molecular studies on populations that have been carefully characterized morphologically.

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